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The effects of down-regulating expression of *Arabidopsis thaliana* membrane-associated acyl-CoA binding protein 2 on acyl-lipid composition☆

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Abstract

Multiple classes of acyl-CoA binding proteins are encoded by plant genomes, including a plant-unique class of predicted integral membrane-proteins. Transcript analysis revealed that both of the integral membrane-acyl-CoA binding proteins of *Arabidopsis thaliana*, ACBP1 and ACBP2, are expressed in all organs. Expression of ACBP2 was highest in developing roots and flowers, and was four-fold greater than expression of ACBP1. Polyclonal antibodies against recombinant ACBP2 specifically recognized a Mr 47k protein that accumulated to similar levels in *A. thaliana* leaves, flowers, and siliques (0.05–0.07% total protein), but was two-fold more abundant in roots. To study the potential role(s) of ACBP2 in acyl-CoA metabolism, expression was down-regulated using hairpin RNA interference (RNAi). Three RNAi lines with at least 70% reduced levels of the ACBP2 protein were analyzed, but displayed no gross alterations in growth or developmental phenotype. A systematic analysis of lipids from developing leaves by electrospray tandem-mass spectrometry revealed little change in the levels of the eight major lipid classes but significant changes in fatty acid composition were observed, particularly for the phospholipids. These data suggest a specialized rather than general role for ACBP2 in plant acyl-lipid metabolism.

Keywords: Acyl-CoA binding protein; Acyl-lipid profiling; Lipid trafficking; *Arabidopsis thaliana*; Hairpin RNAi; MALDI-TOF mass spectrometry

1. Introduction

De novo fatty acid synthesis in plants occurs exclusively within plastids, after which the preponderance of acyl chains are modified and esterified at the endoplasmic reticulum (ER) [1]. Despite this well-known compartmental separation between fatty acid and complex lipid synthesis, the intracellular transport of fatty acids remains a poorly understood aspect of plant lipid metabolism. The fatty acids released from acyl carrier protein at the completion of de novo synthesis are esterified to CoA by outer envelope-localized acyl-CoA synthetases [2,3].

Traffic of acyl-CoAs from the plastids to the ER, or within the cytoplasm, is thought to be facilitated by the soluble low molecular weight acyl-CoA binding proteins (ACBP) that are ubiquitous in eukaryotic cells [4]. Examples of ACBP have been identified in plants, fungi, protozoa and higher animals, and are characterized by an acyl-CoA binding motif [4–7]. Typical plant ACBP are 8–10 kDa and share approximately 60% amino acid identity with the mammalian and yeast orthologs [5,8]. In developing *Brassica napus* seeds these proteins accumulate to 65 μM, 10-fold higher than in any other organ examined [8]. The high levels of ACBP in developing oil-rich seeds imply a role in acyl-CoA transit between plastids and ER for triacylglycerol biosynthesis.

A catalog of the lipid biosynthesis genes from *Arabidopsis thaliana* has been prepared [9,10]. These analyses revealed a single nuclear gene encoding the ACBP, and three additional classes of putative acyl-CoA binding proteins, each of which

☆ The cDNA sequence for ACBP2 described herein has been assigned GenBank Accession AF320561..

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was identified by amino acid-similarity to ACBP within the acyl-CoA binding cleft. The first of these classes includes ACBP1 and 2, integral membrane proteins that are 72% identical at the deduced amino acid sequence level [11–13] and include C-terminal ankyrin repeat motifs [14]. A chimera between ACBP2 and the green fluorescent protein was localized to the plasma membrane [14] where it interacts with an ethylene-responsive element-binding protein through the ankyrin repeat motifs [15]. The other two protein classes include ACBP3, an extracellular protein [16], and ACBP4 and 5, two large cytoplasmic proteins that are 81% identical at the deduced amino acid level [17] and are characterized by C-terminal Kelch domains [18]. Despite the substantial information describing the in vitro characteristics of the recombinant proteins, the in vivo role(s) of the plant acyl-CoA binding proteins remain obscure.

To gain additional insight into the function of the *A. thaliana* acyl-CoA binding proteins, we have employed RNA interference (RNAi) to down-regulate expression of ACBP2. Although this strategy effectively reduced expression in developing leaves, no gross phenotypic changes were observed. In contrast, however, there were significant changes in acyl-lipid composition, particularly within the phospholipids. To our knowledge, this is the first report describing down-regulation of expression of this class of acyl-CoA binding proteins in any plant.

2. Materials and methods

2.1. Plant growth conditions

A. thaliana (ecotype Columbia) plants were grown at 18 °C under low light flux (49 μmol photon m^{-2} s^{-1}) with a 16 h photoperiod. Seeds were sown in a 1:1 mixture of vermiculite and peat moss-enriched soil.

2.2. Heterologous expression of ACBP2 for antibody preparation

A cDNA encoding *A. thaliana* ACBP2 was identified from a developing seed library [19] and was sequenced on both strands prior to submission to GenBank (Accession number AF320561). The open reading frame for was amplified using primers which contained NdeI and XhoI restrictions sites for insertion into the pET28a expression vector (Novagen, Madison, WI). The PCR products were cloned, and sequenced prior to construction of the expression vector. The expression constructs were used to transform *Escherichia coli* strain BL21 (DE3) according to previously described procedures [20]. Proteins were quantified by a dye-binding assay [21] using a kit from Bio-Rad (Hercules, CA).

Recombinant ACBP2 was purified under denaturing conditions in accordance with the manufacturer's instructions (Novagen). For antibody production, purified denatured ACBP2 was dialyzed against 10 mM NaH_2PO_4, pH 7.2, containing 150 mM NaCl, at 4 °C for 16 h. The majority of the recombinant protein precipitated during dialysis. The entire dialysate was concentrated on an ultra-filtration membrane (5000 MW cutoff), then the insoluble material was homogenized by passing through a 22 gauge syringe point prior to use as an antigen (Cocalico Antibodies Inc., Reamstown, PA).

2.3. Analysis of recombinant ACBP2 by mass spectrometry

Tryptic peptides were prepared by in-gel digestion of recombinant ACBP2, as described by Shevchenko et al. [22]. Equal volumes of tryptic peptides and α-cyano-4-hydroxycinnamic acid (Sigma Chemical Company, St. Louis, MO) were spotted onto a stainless steel MALDI target plate. Peptide standards (Applied Biosystems, Foster City, CA) were spotted in close proximity to samples for calibration. Peptide masses were determined using a Delayed Extraction Matrix Assisted Laser Desorption Ionization Time of Flight mass spectrometer (Voyager DE-PRO MALDI-TOF, Applied Biosystems). Spectra were acquired in positive ion delayed extraction reflector mode for highest resolution and mass accuracy.

2.4. Immunoblot analysis of ACBP2 in Arabidopsis thaliana

To quantify the ACBP2 protein in samples from *A. thaliana*, roots, rosette leaves (14-and 40-days after germination), flowers, and developing siliques (8–14 days after flowering) were harvested from soil-grown plants. Fresh material (1–5 mg) was homogenized in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT) using a Dounce homogenizer. Equal amounts of protein (50 μg) were resolved by SDS-PAGE, then electroblotted to nitrocellulose membranes which were probed with antibodies as previously described [23].

2.5. Quantification of transcript abundance

A total RNA fraction was isolated from fresh plant material using a guanidine thiocyanate extraction protocol [24], and quantified by absorbance at 260 nm. Resolution of RNA was achieved by electrophoresis in 0.8% (w/v) agarose gels containing 2.2 M formaldehyde. The RNA was transferred to Nytran membrane (Amersham Biosciences, Piscataway, NJ), then fixed by heating at 80 °C for 3 h under vacuum. Ribosomal RNA was visualized by staining the blots with methylene blue, then destained with nuclease-free water. Radioactive probes were generated by random primer labeling (RediPrime II labeling kit, Amersham Biosciences) using full-length cDNAs as templates. The specific activities of the ACBP1 and 2 probes differed by less than 10%. Pre-hybridization, hybridization, and washing were performed as described previously [24]. Double stranded DNA for each isoform (1 ng) was used to determine the extent of cross-hybridization. The signals were quantified from 12-bit TIFF images of autoradiographs using ImageQuant densitometric software (Amersham Biosciences). All values were corrected by subtracting the local background.

2.6. Construction of the ACBP2 RNAi vector and plant transformation

A 120 bp fragment of the *A. thaliana* ACBP2 reading frame, bases 448 to 567 of GenBank Accession number AF320561, was amplified using primers: 5′-ggatcctcagccatcagctct and 5′-gaattcgtaaagctgagtgacaa which include BamHI and EcoRI sites, respectively. The ACBP2 amplicon was cloned in opposing orientations around intron one of the *A. thaliana* FAD2 gene, to facilitate optimal hairpin formation [25]. Expression of the hairpin cassette was driven by the 35S CMV promoter and halted using an octopine synthase terminator (Fig. 3B). The entire expression cassette was cloned into a binary expression vector downstream of and in the same orientation as a phosphinothricin-N-acetyltransferase gene cassette, to allow selection of transformants by resistance to the herbicide glufosinate.

A. thaliana plants were transformed with the ACBP2 RNAi vector by floral dip [26]. Transformants were selected by sowing seeds in soil saturated with 0.005% (v/v) glufosinate, followed by foliar application of a 0.029% solution of glufosinate 10 days after germination. Fifteen first generation (T1) plants resistant to glufosinate were screened for ACBP2 expression by immunoblot analyses. Twelve second generation (T2) lines containing reduced levels of the ACBP2 protein were subjected to an additional round of glufosinate treatment to verify transformation. The three independent T3 lines that consistently contained the lowest levels of ACBP2 protein were further studied.

2.7. Lipid analysis

Homozygous T3 lines 1–1, 4–7, and 6–26 were sown beside wild type plants; no glufosinate herbicide selection was applied to avoid potential herbicide effects on fatty acid composition. Lipids were extracted from developing leaves of similar size (6–9 mg FW) immediately as described by Welti et al. previously [27]. Extracts were evaporated to dryness under N_2, then the lipids analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Kansas State Lipidomics facility (http://www.k-state.edu/lipid/lipidomics/) using a triple quadrupole mass spectrometer [27].

Developing roots (10 to 27 mg FW), leaves (23 to 35 mg FW), and siliques (22 to 33 mg FW) were individually pooled from at least four different 4-week old plants. Samples were refluxed in Teflon-lined screw cap glass vials with 10% BCl_3 in methanol at 95 °C for 1 h with vortexing at 10 min intervals; heptadecanoic acid (4% of FW, dissolved in toluene) was used as the internal standard. Fatty acid methyl esters were extracted with hexane and separated using a DB-23 column (30 m, 0.25 mm i.d., 0.25 μm film, Agilent) for separation with an Agilent 6890 gas chromatograph. He was used as the carrier, in split mode (30:1) at a split flow of 30 mL min^{-1} and a total flow of 33.9 mL min^{-1}. The oven temperature was increased from 150 °C to 200 °C at 2 °C min^{-1} with a 5 min final hold. Samples were analyzed in tandem using both mass selective and flame ionization detectors for analyte identification and quantification, respectively. The makeup gas for FID was N_2.

3. Results

3.1. Analysis of recombinant A. thaliana ACBP2 by mass spectrometry

Under a wide range of experimental conditions, *A. thaliana* ACBP2 was insoluble when expressed in *E. coli*. Attempts to solubilize the recombinant protein were unsuccessful; at urea concentrations of less than 1.0 M ACBP2 precipitated. The protein was subsequently purified under denaturing conditions by binding to the Ni-NTA matrix and elution with imidazole. The final preparations were >95% pure as judged by Coomassie blue-staining of SDS-gels (Fig. 1).

Induction of full-length ACBP2 (which additionally contains a 20 residue N-terminal extension that includes a His_6-tag)

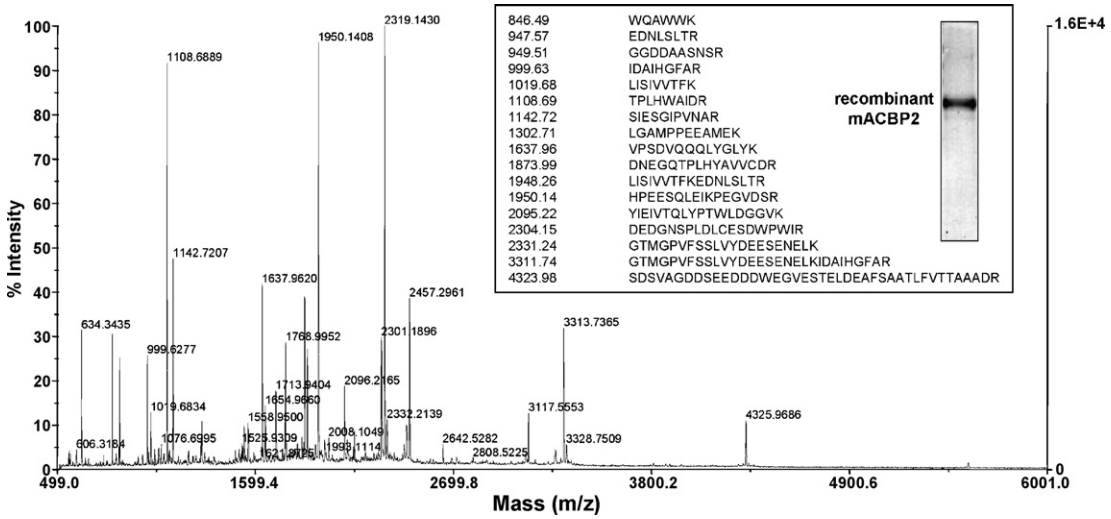

Fig. 1. Mass spectrum of peptides obtained from purified ACBP2. The recombinant protein was resolved by SDS-PAGE (inset panel), then digested in-gel with trypsin. The resultant peptides were analyzed using a Voyager DE-PRO MALDI-TOF mass spectrometer. The inset panel shows the monoisotopic masses of peptides and the corresponding sequence match. Approximately 66% of the deduced sequence was covered by the observed ions.

yielded a Mr 52k polypeptide. This is substantially larger than the size deduced from the cDNA sequence. The identity of the Mr 52k protein was determined by peptide mass fingerprint analysis using MALDI-TOF MS. Analysis of tryptic peptides in the range 500 to 6000 m/z yielded 24 major ions, 17 of which could be unambiguously assigned to recombinant ACBP2 for a total of 66% sequence coverage (Fig. 1). This verified the identity of the Mr 52k protein as ACBP2. The atypical electrophoretic mobility is likely due at least in part to the hydrophobic α-helical sequence that is characteristic of this class of acyl-CoA binding proteins.

3.2. Analysis of ACBP2 expression

Northern analyses revealed that the ACBP2 transcript was at least four-fold more abundant than ACBP1 (Fig. 3A). The ACBP2 transcript was observed in roots, leaves, flowers, and siliques, and accumulated at approximately 40–50% higher levels in roots and flowers than in leaves and siliques. The transcript for ACBP1 was also detected in all four organs, where siliques and flowers contained 10–20% higher levels than roots and leaves. No cross-hybridization was observed between the ACBP1 and 2 DNA probes under the conditions employed.

Since ACBP2 transcript was expressed at higher levels than ACBP1, polyclonal antibodies were produced for subsequent biochemical analysis. Antibodies from two different immunization regimens recognized the Mr 52k ACBP2 polypeptide from whole bacterial lysates (Fig. 2A). These antibodies detected a faintly expressed Mr 47k protein in 14 days *A. thaliana* total leaf protein samples while pre-immune sera did not give any reaction (Fig. 2B). The Mr of this protein is 9k larger than the MW deduced from the primary sequence (38,480 Da). An additional Mr 61k band was observed, however, this band had a diffuse appearance and cross-reacted with secondary antibody controls indicating non-specific binding. Based upon comparisons with recombinant protein standards, ACBP2 was expressed at approximately 0.05% to 0.07% of the total protein. This Mr 47k protein was also detected in flowers and siliques, and was two-fold more abundant in roots than in any other organ tested (Fig. 2C).

3.3. Intron-spliced hairpin RNA silencing of ACBP2 results in subtle changes in lipid composition

To down-regulate expression of ACBP2 in *A. thaliana*, a hairpin RNAi vector was constructed using 120 bp of the ACBP2 ORF (Fig. 3B). The 120 bp region was cloned in opposing orientations around intron 1 from the *A. thaliana* FAD2 gene as described previously [25]. The 35S CaMV promoter was used to drive expression of this hairpin transcript (Fig. 3B). Immunoblot analysis of developing leaves from wild type *A. thaliana* and three RNAi lines (1–1, 4–7 and 6–26) demonstrate reduced expression of the Mr 47k polypeptide. However, no gross growth or development phenotype was observed for any of the RNAi lines. Down-regulation of ACBP2 expression, normalized to RuBisCO LSU expression, was approximately 85% in the three RNAi lines analyzed (Fig. 2D).

Developing leaves from the three RNAi lines were analyzed for acyl-lipid content in triplicate by LC-MS/MS. Compositional

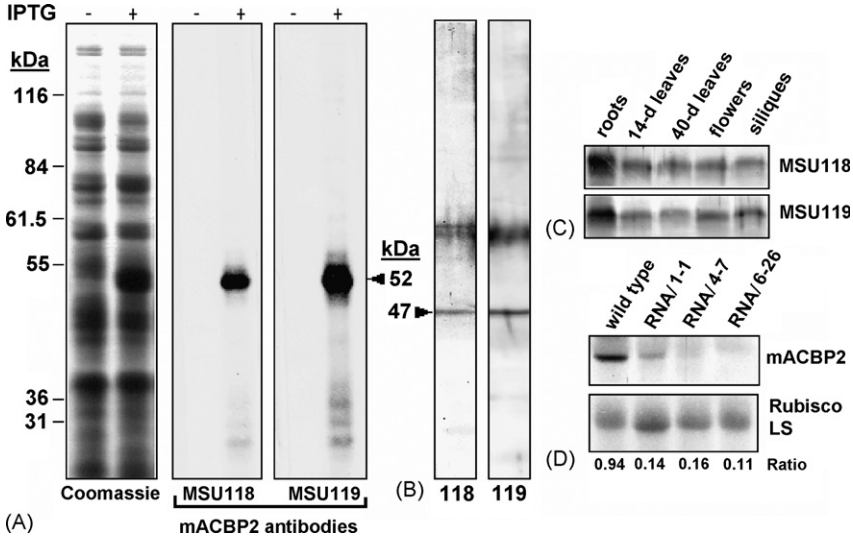

Fig. 2. Immunoblot analysis of the *A. thaliana* ACBP2 protein. (A) Total *E. coli* lysate (20 μg protein) from un-induced or induced cells were resolved by SDS-PAGE and stained with Coomassie blue. For immunoblot analyses 50 ng of total protein were resolved for analysis with antisera MSU118 or MSU119; (B) total *A. thaliana* wild type leaf proteins (10 μg) were resolved by SDS-PAGE, blotted to nitrocellulose and probed with anti-ACBP2 antiserum. Both antisera decorated a Mr 47k protein; (C) immunoblot analysis of ACBP2 expression in developing roots, 14-day leaves, 40-day leaves, flowers, and developing siliques from wild type *A. thaliana*. Whole organs were pulverized directly in SDS-PAGE sample buffer using a Dounce homogenizer. The homogenates were clarified by centrifugation, and supernatants taken for protein quantification. Equal amounts of protein (50 μg) were analyzed from each organ; (D) immunoblot analysis of ACBP2 expression in wild type and RNAi lines of *A. thaliana*. Developing rosette leaves from wild type and RNAi lines were homogenized in sample buffer and 50 μg of protein resolved by SDS-PAGE. Blots were probed with MSU119 antiserum. To evaluate sample loading, replicate blots were probed with polyclonal antibodies to RuBisCO LSU. Down-regulation of ACBP2 was quantified using the ImageQuant TL v2003 software (Amersham Biosciences). The volume of the ACBP2 protein band was normalized using the volume of the RuBisCO LSU band.

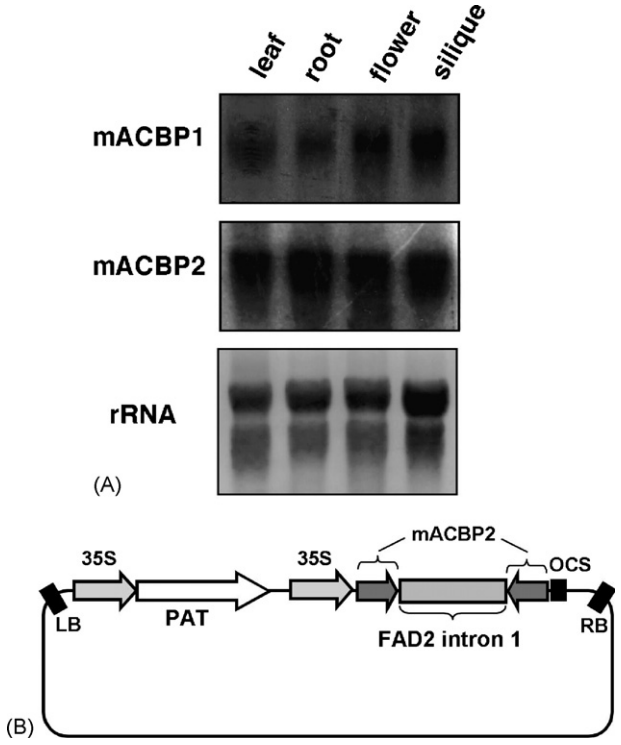

Fig. 3. Analysis of transcript abundance in *A. thaliana*. (A) Total RNA (20 μg) isolated from developing leaves, roots, flowers, or siliques was resolved by electrophoresis in 0.8 agarose gels containing 2.2 M formaldehyde. Probes for ACBP1 and 2 were of similar specific activity (<10% difference), and minimal cross-hybridization was observed under the conditions employed. Methylene blue-stained rRNA bands are shown as loading controls. The data shown are representative of two independent northern blots; (B) diagram of RNAi construct used for silencing ACBP2 expression. The phosphinothricin acetyltransferase (PAT) resistance gene and RNA interference cassettes were both expressed with a 35S promoter and terminated with an octopine synthase (OCS) terminator. The RNAi cassette contained a 120 bp region of the ACBP2 ORF in opposing orientations of the *A. thaliana* FAD2 intron 1 to facilitate hairpin formation.

analyses revealed no statistically significant changes in levels of the eight predominant lipid classes between wild type and RNAi plants (Table 1). However, acyl chain composition of the six most abundant lipid classes revealed reproducible and statistically significant changes (Table 2). In general, phospholipid species exhibited more changes than galactolipids. Each of the four major phospholipids contained a higher percentage of 34:2 (18:2 + 16:0) at the expense of 34:3 for phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), and 34:4 for phosphatidylglycerol (PG). Percent composition of 36:4 and 36:5 PC and PE lipid species also increased while 36:6 decreased. A subtle change was also observed in MGDG; the mole percentage of the 34:6 species was higher while the 36:6 lipid species was lower.

Although informative, lipidomic analysis using LC-MS/MS does not give an all-inclusive assessment of the total fatty acid-composition due to the inability to resolve acyl-chain positional composition. To determine if the changes in acyl-composition among phospholipid classes were sufficient to alter total membrane characteristics, fatty acids were transmethylated from whole organs and analyzed by gas chromatography. *A. thaliana* leaves contain eight prominent fatty acids, and although changes were apparent in $16{:}1^{\Delta 9}$, 16:3, $18{:}1^{\Delta 9}$, and 18:2 fatty acids, compared to wild type plants, the changes were subtle and not observed in all three RNAi lines (Table 3). Since ACBP2 was detected in all organs, but was particularly abundant in roots, this organ, along with siliques, was also analyzed for fatty acid composition. In siliques, only minor compositional changes were observed; lines 1–1 and 4–7 both had reduced $18{:}1^{\Delta 9}$ and 18:2, and slightly increased levels of 20:3 and 22:0 fatty acids. Line 1–1 also had lower percentages of 18:3 and $20{:}1^{\Delta 11}$. *A. thaliana* roots contained six major fatty acids, and in all RNAi lines the percentage of 18:2 decreased by 5–10% while 18:3 increased 10–15%.

4. Discussion

In plants, de novo fatty acid biosynthesis occurs exclusively in the plastids, though acyl-chains are utilized in every cellular compartment. Additionally, some organs, tissues, and membranes have unique acyl-chain requirements. For instance, epidermal cells require acyl-chains for cuticular wax production [28], and developing seeds require large quantities of fatty

Table 1
Lipid composition of rosette leaves (2–3 weeks after flowering) from *A. thaliana* wild type and three RNAi lines with reduced levels of the ACBP2 protein

	Wild type (mol%)	RNAi 1–1 (mol%)	RNAi 4–7 (mol%)	RNAi 6–26 (mol%)
DGDG	23.9 (1.7)	22.0 (1.6)	22.4 (0.8)	22.8 (4.0)
MGDG	50.3 (8.9)	52.9 (2.9)	49.5 (3.2)	51.5 (11.4)
PG	9.1 (1.5)	8.5 (0.7)	8.5 (0.9)	8.9 (0.8)
PC	6.9 (0.9)	7.2 (0.2)	8.2 (2.0)	7.1 (1.7)
PE	6.4 (0.3)	6.1 (0.4)	7.2 (0.8)	6.1 (0.7)
PI	2.4 (0.5)	2.1 (0.1)	2.6 (0.6)	2.2 (0.7)
PS	0.46 (0.08)	0.48 (0.03)	0.56 (0.16)	0.52 (0.19)
PA	0.58 (0.04)	0.51 (0.13)	0.92 (0.38)	0.71 (0.19)
lysoPG	0.004 (0.003)	0.006 (0.004)	0.015 (0.009)	0.014 (0.008)
lysoPC	0.030 (0.010)	0.030 (0.004)	0.038 (0.009)	0.031 (0.007)
lysoPE	0.059 (0.004)	0.055 (0.009)	0.063 (0.005)	0.057 (0.004)

Lipids were quantified by electrospray-ionization MS/MS using combinations of precursor and neutral loss scans as mass signatures for lipid head groups as described previously [27]. Lipid concentrations are expressed as mole percentages and each value represents the mean of three biological repetitions (i.e., independent leaf extractions). Standard deviations are indicated in parentheses. *Abbreviations*: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid.

Table 2
Determination of fatty acid composition of lipid classes from rosette leaves of wild type (wt.) and RNAi ACBP2 lines (1–1, 4–7, 6–26) of *Arabidopsis thaliana* using LC–MS/MS

	MGDG (mol%)				DGDG (mol%)				PG (mol%)			
	wt.	1–1	4–7	6–26	wt.	1–1	4–7	6–26	wt.	1–1	4–7	6–26
32:0	–	–	–	–	–	–	–	–	1.48	1.38	2.06	1.87
32:1	–	–	–	–	–	–	–	–	6.70	6.50	6.97	6.75
34:0	–	–	–	–	–	–	–	–	0.08	0.16	0.27	0.11
34:1	0.17	0.64	0.25	0.31	0.84	1.10	0.79	1.29	5.15	7.46	7.62	5.57
34:2	0.14	0.35	0.20	0.25	1.70	3.11	1.87	2.34	**11.77**	**14.52**	**14.27**	**14.92**
34:3	1.87	1.11	1.73	1.30	**20.16**	**17.17**	**17.60**	**17.76**	26.02	24.98	28.72	27.52
34:4	0.92	1.71	1.37	1.86	0.71	0.82	0.91	1.13	**48.81**	**45.00**	**40.07**	**43.26**
34:5	–	–	–	–	0.42	0.92	0.74	0.76	–	–	–	–
34:6	**60.63**	**70.57**	**63.46**	**69.61**	2.50	4.47	3.19	4.06	–	–	–	–
36:1	0.08	0.11	0.00	0.05	0.00	0.00	0.01	0.04	–	–	–	–
36:2	–	–	–	–	0.02	0.01	0.08	0.06	–	–	–	–
36:3	0.01	0.02	0.11	0.03	1.34	1.32	1.12	1.25	–	–	–	–
36:4	0.50	0.74	0.54	0.72	1.11	1.74	2.00	1.78	–	–	–	–
36:5	0.00	1.08	0.37	0.36	–	–	–	–	–	–	–	–
36:6	**35.22**	**23.21**	**31.40**	**25.22**	70.47	68.72	70.60	68.77	–	–	–	–
38:2	–	–	–	–	–	–	–	–	–	–	–	–
38:3	0.36	0.42	0.43	0.18	–	–	–	–	–	–	–	–
38:4	0.08	0.03	0.11	0.11	0.04	0.05	0.04	0.05	–	–	–	–
38:5	–	–	–	–	–	–	–	–	–	–	–	–
38:6	0.00	0.00	0.03	0.00	0.69	0.55	1.02	0.73	–	–	–	–
40:2	–	–	–	–	–	–	–	–	–	–	–	–
40:3	–	–	–	–	–	–	–	–	–	–	–	–
42:2	–	–	–	–	–	–	–	–	–	–	–	–
42:3	–	–	–	–	–	–	–	–	–	–	–	–
42:4	–	–	–	–	–	–	–	–	–	–	–	–

	PC (mol%)				PE (mol%)				PI (mol%)			
	wt.	1–1	4–7	6–26	wt.	1–1	4–7	6–26	wt.	1–1	4–7	6–26
32:0	0.06	0.07	0.08	0.06	–	–	–	–	–	–	–	–
32:1	–	–	–	–	–	–	–	–	–	–	–	–
34:0	–	–	–	–	–	–	–	–	–	–	–	–
34:1	0.66	0.86	1.08	0.84	–	–	–	–	0.20	0.00	0.25	0.00
34:2	**9.43**	**13.12**	**12.46**	**12.32**	**15.62**	**24.33**	**19.49**	**20.97**	**18.58**	**29.76**	**25.22**	**28.43**
34:3	**40.51**	**31.32**	**36.51**	**33.43**	**47.43**	**36.45**	**42.30**	**39.61**	**74.45**	**62.96**	**66.80**	**63.36**
34:4	0.44	0.61	0.56	0.68	0.32	0.38	0.36	0.42	0.03	0.02	0.22	0.17
34:5	–	–	–	–	–	–	–	–	–	–	–	–
34:6	–	–	–	–	–	–	–	–	–	–	–	–
36:1	0.06	0.02	0.04	0.06	0.01	0.00	0.01	0.02	0.06	0.29	0.16	0.21
36:2	1.31	1.93	1.49	1.79	1.60	2.35	1.65	2.13	0.00	0.48	0.71	0.66
36:3	5.51	4.87	4.88	4.81	3.90	3.59	3.52	3.55	1.60	1.45	1.63	1.34
36:4	**4.54**	**7.98**	**6.80**	**7.43**	**4.97**	**8.33**	**7.02**	**7.51**	0.51	0.51	0.51	0.74
36:5	**15.17**	**20.50**	**17.00**	**18.84**	**11.34**	**13.36**	**12.87**	**12.96**	**0.89**	**1.78**	**1.25**	**1.72**
36:6	**21.30**	**17.71**	**18.19**	**18.68**	**10.96**	**7.15**	**8.97**	**8.64**	3.67	2.75	3.25	3.36
38:2	0.10	0.15	0.13	0.13	0.25	0.37	0.31	0.28	–	–	–	–
38:3	0.36	0.33	0.32	0.34	0.47	0.47	0.50	0.47	–	–	–	–
38:4	0.16	0.18	0.15	0.16	0.07	0.15	0.08	0.11	–	–	–	–
38:5	0.13	0.16	0.11	0.16	0.16	0.16	0.18	0.19	–	–	–	–
38:6	0.17	0.13	0.12	0.15	0.20	0.16	0.16	0.20	–	–	–	–
40:2	0.03	0.02	0.03	0.03	0.36	0.68	0.53	0.63	–	–	–	–
40:3	0.04	0.02	0.04	0.05	0.46	0.44	0.48	0.46	–	–	–	–
42:2	–	–	–	–	0.68	0.82	0.64	0.83	–	–	–	–
42:3	–	–	–	–	0.87	0.59	0.70	0.77	–	–	–	–
42:4	–	–	–	–	0.32	0.21	0.25	0.26	–	–	–	–

The data are mean mole percent values of three biological replicates. Standard deviations for most fatty acid species were less than 10% of the mean values. Fatty acids below detection limits are noted with a hyphen. Values for ACBP2 RNAi lines that are statistically different from wild type (t-test probability 95%) are in bold typeface. For clarity, phosphatidylserine and phosphatidic acid composition data were omitted.

Table 3
Analysis of fatty acid methyl esters from siliques, leaves, and roots of wild type and RNAi ACBP2 lines of *Arabidopsis thaliana*

	16:0	$16:1^{\Delta 9}$	$16:1^{\Delta 3}$	16:3	18:0	$18:1^{\Delta 9}$	$18:1^{\Delta 11}$	18:2	18:3	20:0	$20:1^{\Delta 11}$	$20:1^{\Delta 13}$	20:2	20:3	22:0	22:1	Other
Siliques																	
Wild type	10.5	1.2	–	–	3.9	10.7	1.9	27.1	20.1	2.1	12.0	1.4	1.7	0.5	4.1	1.4	1.4
RNAi 1–1	11.3	1.3	–	–	4.1	9.7	2.1	25.5	18.7	1.7	10.5	1.4	1.2	1.5	7.2	1.1	3.4
RNAi 4–7	10.5	1.5	–	–	4.1	9.7	1.7	25.9	20.6	2.2	12.1	1.3	1.7	0.7	4.7	1.4	1.9
RNAi 6–26	10.1	1.0	–	–	3.8	11.0	2.0	27.1	19.7	2.2	12.6	1.4	1.6	0.7	3.9	1.3	1.4
Leaves																	
Wild type	13.4	3.3	1.0	11.1	2.4	2.5	–	7.1	59.2	–	–	–	–	–	–	–	–
RNAi 1–1	14.3	2.3	2.4	9.3	2.7	2.2	–	6.2	62.0	–	–	–	–	–	–	–	–
RNAi 4–7	14.2	2.6	1.2	10.0	2.5	3.5	–	8.7	57.3	–	–	–	–	–	–	–	–
RNAi 6–26	13.7	2.8	1.2	11.1	2.0	3.4	–	6.5	59.4	–	–	–	–	–	–	–	–
Roots																	
Wild type	23.0	2.2	–	–	6.7	6.3	–	44.3	17.5	–	–	–	–	–	–	–	–
RNAi 1–1	21.8	2.1	–	–	4.8	4.7	–	42.7	23.8	–	–	–	–	–	–	–	–
RNAi 4–7	23.1	3.7	–	–	6.5	6.0	–	38.1	22.7	–	–	–	–	–	–	–	–
RNAi 6–26	23.1	1.7	–	–	8.0	7.0	–	40.0	20.2	–	–	–	–	–	–	–	–

The data are mean mole percent values of three biological replicates. Standard deviations for most fatty acid species were less than 15% of the mean values. Fatty acids below detection limits are noted with a hyphen.

acids for storage lipid accumulation (reviewed in ref. [29]). Opposing synthesis are acyl-chain turnover and catabolism, which require the β-oxidation pathway for conversion of acyl-chains to acetyl-CoA [30]. Furthermore, some physiological or developmental events require remodeling of the acyl-chain composition of some membranes [31,32]. For each of these processes acyl-chain metabolism requires esterification to CoA. Thus synthesis, modification, and degradation of lipids in plant cells require a complex network of transport to facilitate the traffic of acyl-CoA. This complexity is exemplified in the *A. thaliana* genome where nine acyl-CoA synthetase genes have been identified [33], including those encoding plastidial [2] and peroxisomal [34] enzymes.

The soluble, low molecular weight ACBP has been observed in all four eukaryotic kingdoms, and 11 species of eubacteria, suggesting a conserved role in acyl-CoA trafficking [4]. The ACBP is abundant in developing plant organs, particularly in developing *Brassica* seeds when fatty acid biosynthesis is at a maximum [5,8]. The levels of ACBP have been reported as high as 65 μM in developing seeds and 1 μM in developing leaves of *A. thaliana* and *B. napus* [8].

In addition to ACBP, there are plant-unique genes encoding multiple families of larger and more structurally elaborated acyl-CoA binding proteins [11–13,15–17,35,36]. The *A. thaliana* genome encodes three additional classes of acyl-CoA binding proteins. Both ACBP1 (At5g53470) and ACBP2 (At4g27780) are integral membrane proteins with characteristic C-terminal ankyrin repeat protein interaction domains [11–13,15,35]. The ACBP3 protein (At4g24230) has a N-terminal signal sequence which directs it into the lumen of the ER and ultimately to the periplasm/extracellular matrix [16]. The ACBP4 (At3g054420) and ACBP5 (At5g27630) proteins are cytoplasmic and have characteristic C-terminal Kelch domains that mediate protein interactions [17]. All of these recombinant proteins can bind oleoyl-CoA and palmitoyl-CoA, and are thus bona fide acyl-CoA binding proteins.

The results from our Northern analyses indicate that ACBP1 and 2 genes are expressed in all major plant organs, suggesting these proteins play a central, fundamental-role in metabolism. However, the ACBP2 protein accumulated to a relatively higher level in roots indicating an additional or more specific role in this organ. The results from quantitative analyses indicate that the ACBP2 transcript is approximately four-fold more abundant than that of ACBP1 in roots. It has previously been proposed that ACBP2 is, at least in part, involved in epidermal wax production, based upon the plasma membrane-localization [35]. To gain further insight, ACBP2 was chosen as the candidate for gene-silencing because it is the predominant form. Using hairpin RNAi to down-regulate ACBP2 expression resulted in reduction in protein levels by 50–85% in T1 plants, compared with wild type levels. Despite the extensive reduction in expression, none of 15 T1 lines analyzed showed any gross alteration in growth or developmental phenotype. This suggests that either ACBP2 is not essential for the bulk flow of acyl-CoAs from the plastid to the ER, as this would likely have had a dramatic phenotypic impact, or that plants are somehow able to compensate for down-regulation of ACBP2 expression.

The relationship between ACBP2 and acyl-CoA metabolism was evaluated by analyzing the lipid content of the three RNAi lines that had the greatest decrease in protein levels. The metabolic profiling of leaf lipids in these mutants revealed no change in the sum composition of galactolipid or phospholipid classes when compared to wild type (Table 1). The acyl-chain composition of some phospholipid classes, however, was altered from wild type values (Table 2). These differences were observed in each of the three independent ACBP2 RNAi lines. The homozygous RNAi plants were sown at the same time as wild type plants and were not sprayed with glufosinate, so these changes are not be due to herbicide selection or developmental differences. It is also unlikely that the changes in acyl-chain composition are a consequence of transformation; the same acyl-group and lipid classes were altered in each of the three independent lines that were each analyzed in triplicate.

Quantitative analysis of acyl-lipids using MS/MS is a recently developed technique that has been validated for analysis of *A. thaliana* leaves [27]. Analysis of lipids from wild type *A. thaliana* resulted in lipid composition levels similar to those recently reported using thin-layer chromatography separation methods [37]. However, the percentages of MGDG and DGDG were approximately 15% and 4% higher, respectively, compared to TLC quantification. This difference could be because the prevalent plant sulfolipid, sulfoquinovosyldiacylglycerol, is not quantifiable using current MS/MS. Also, the phospholipids PC, PG, PE, PI, and PS were approximately 4%, 3%, 3%, 3%, and 0.4%, respectively, lower in this study compared to [37]. This could be due to differences in lipid extraction procedures, since levels of each of the polar lipids were lower in this investigation. Despite these small differences, the order of abundance for each lipid class is comparable (i.e., MGDG $\gg$ DGDG $\gg$ PG $>$ PC) between these investigations. Furthermore, the acyl-composition within each lipid class is comparable (Table 2).

In general, silencing of ACBP2 influenced the acyl-chain composition of phospholipids more than galactolipids. Specifically, the 34:2, 36:4, and 36:5 phospholipids were higher while 34:3, 34:4, and 36:6 lipids were lower. These small but reproducible changes observed within lipid classes did not appreciably influence the overall fatty acid composition (Table 3). This was not unexpected since the acyl-CoA binding proteins are involved with transport, not synthesis of fatty acids. Furthermore, it has been suggested that plants are generally resistant to changes in fatty acid composition as this can often cause deleterious side effects due to membrane restructuring [37–41]. If ACBP2 is involved in acyl-CoA transport for anabolic reactions, the changes in fatty acid composition suggest a slight preference for palmitoyl-CoA due to the increase in 34:2 phospholipids (Table 2) and 16:0 (Table 3) within leaves. These data are in agreement with the substrate specificity of ACBP2 observed previously [12].

Overall, the subtle changes in lipid composition seen in plants with reduced ACBP2 expression suggest that this protein is not critical for bulk intracellular transport of acyl-CoAs in *A. thaliana*. Thus, a more specialized role for this membrane-bound class of ACBPs is likely. Palmitoyl-CoA is the immediate substrate for serine palmitoyltransferase, a key enzyme in sphingolipid biosynthesis [31,42,43]. Perhaps ACBP2 is involved in the transport of acyl-chains for sphingoid base and sphingolipid synthesis, a possibility we are currently investigating. Another possibility is that ACBP2 has an involvement in synthesis of protein glycosylphosphatidylinositol-membrane anchors [32]. Most glycosylphosphatidylinositol-anchored proteins are localized at the plasma membrane, and many are expressed specifically in roots [44,45].

It is not uncommon to observe fractional down-regulation with RNAi approaches [46,47]. One possible explanation for partial silencing is tissue-specific expression of the target gene [48]. If, for example, ACBP2 were targeted for epidermis-specific expression, the 35S promoter would not provide optimal expression of the intron-interrupted hairpin transcript in this cell type. Alternatively, down-regulating ACBP2 expression might be compensated for by up-regulating ACBP1, or some other ACBP, particularly if complete gene silencing has profound negative consequences. Although no gross growth or developmental phenotypic consequences were observed with the RNAi lines for ACBP2, it should be taken into account that silencing was fractional and that only a single set of environmental conditions was considered. Possibly a complete knockout of expression, or plant growth under different conditions of light, temperature, nutrition, etc., will reveal phenotypic consequences. Towards this end, we have recently identified multiple allelic T-DNA gene insertions ACBP2 and are in the process of producing homozygous lines.

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References

[1] J.B. Ohlrogge, J. Browse, Lipid biosynthesis, Plant Cell 7 (1995) 957–970.
[2] J.A. Schnurr, J.M. Shockey, G.-J. de Boer, J.A. Browse, Fatty acid export from the chloroplast. Molecular characterization of a major plastidial acyl-coenzyme A synthetase from *Arabidopsis thaliana*, Plant Physiol. 129 (2002) 1700–1709.
[3] M. Ferro, D. Salvi, S. Brugiere, S. Miras, S. Kowalski, M. Louwagie, J. Garin, J. Joyard, N. Rolland, Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*, Mol. Cell. Proteomics 2 (2003) 325–345.
[4] M. Burton, T.M. Rose, N.J. Faergeman, J. Knudsen, Evolution of the acyl-CoA binding protein (ACBP), Biochem. J. 392 (2005) 299–307.
[5] M.J. Hills, R. Dann, D. Lydiate, A. Sharpe, Molecular cloning of a cDNA from *Brassica napus* L. for a homologue of acyl-CoA-binding protein, Plant Mol. Biol. 25 (1994) 917–920.
[6] B. Gaigg, T.B. Neergaard, R. Schneiter, J.K. Hansen, N.J. Faergeman, N.A. Jensen, J.R. Andersen, J. Friis, R. Sandhoff, H.D. Schroder, J. Knudsen, Depletion of acyl-coenzyme A-binding protein affects sphingolipid synthesis and causes vesicle accumulation and membrane defects in *Saccharomyces cerevisiae*, Mol. Biol. Cell 12 (2001) 1147–1160.
[7] K.G. Milne, M.A. Ferguson, Cloning, expression, and characterization of the acyl-CoA-binding protein in African trypanosomes, J. Biol. Chem. 275 (2000) 12503–12508.
[8] N.J. Engeseth, R.S. Pacovsky, T. Newman, J.B. Ohlrogge, Characterization of an acyl-CoA-binding protein from *Arabidopsis thaliana*, Arch. Biochem. Biophys. 331 (1996) 55–62.
[9] S. Mekhedov, O. Martinez de Ilarduya, J.B. Ohlrogge, Toward a functional catalog of the plant genome: a survey of genes for lipid biosynthesis, Plant Physiol. 122 (2000) 389–401.
[10] F. Beisson, A.J.K. Koo, S. Ruuska, J. Schwender, M. Pollard, J.J. Thelen, T. Paddock, J.J. Salas, L. Savage, A. Milcamps, V. Mhaske, Y. Cho, J.B. Ohlrogge, *Arabidopsis thaliana* genes involved in acyl lipid metabolism: a 2003 census of the candidates, a study of the distribution of ESTs in tissues and a web-based database, Plant Physiol. 132 (2003) 681–697.
[11] M.-L. Chye, *Arabidopsis thaliana* cDNA encoding a membrane-associated protein with an acyl-CoA binding domain, Plant Mol. Biol. 38 (1998) 827–838.

[12] M.-L. Chye, H.-Y. Li, M.-H. Yung, Single amino acid substitutions at the acyl-CoA-binding domain interrupt 14[C] palmitoyl-CoA binding of ACBP2, an *Arabidopsis thaliana* acyl-CoA-binding protein with ankyrin repeats, Plant Mol. Biol. 44 (2000) 711–721.
[13] H.-Y. Li, M.-L. Chye, Membrane localization of *Arabidopsis thaliana* acyl-CoA binding protein ACBP2, Plant Mol. Biol. 51 (2003) 483–492.
[14] P. Bork, Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally, Proteins 17 (1993) 363–374.
[15] H.-Y. Li, M.-L. Chye, Arabidopsis Acyl-CoA-binding protein ACBP2 interacts with an ethylene-responsive element-binding protein, AtEBP, via its ankyrin repeats, Plant Mol. Biol. 54 (2004) 233–243.
[16] K.C. Leung, H.Y. Li, S. Xiao, M.H. Tse, M.L. Chye, Arabidopsis ACBP3 is an extracellularly targeted acyl-CoA-binding protein, Planta 223 (2006) 871–881.
[17] K.C. Leung, H.Y. Li, G. Mishra, M.L. Chye, ACBP4 and ACBP5, novel Arabidopsis acyl-CoA-binding proteins with kelch motifs that bind oleoyl-CoA, Plant Mol. Biol. 55 (2004) 297–309.
[18] J. Adams, R. Kelso, L. Cooley, The Kelch repeat superfamily of proteins: propellers of cell function, Trends Cell Biol. 10 (2000) 17–24.
[19] J.A. White, J. Todd, T. Newman, N. Focks, T. Girke, O.M. de Ilarduya, J.G. Jaworski, J.B. Ohlrogge, C. Benning, A new set of *Arabidopsis thaliana* expressed sequence tags from developing seeds. The metabolic pathway from carbohydrates to seed oil, Plant Physiol. 124 (2003) 1582–1594.
[20] J.J. Thelen, M.G. Muszynski, N.R. David, M.H. Luethy, T.E. Elthon, J.A. Miernyk, D.D. Randall, The dihydrolipoamide *S*-acetyltransferase subunit of the mitochondrial pyruvate dehydrogenase complex from maize contains a single lipoyl domain, J. Biol. Chem. 274 (1999) 21769–21775.
[21] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[22] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels, Anal. Chem. 68 (1996) 850–858.
[23] J.J. Thelen, S. Mekhedov, J.B. Ohlrogge, *Brassicaceae* express multiple isoforms of biotin carboxyl carrier protein in a tissue-specific manner, Plant Physiol. 125 (2001) 2016–2028.
[24] Preparation and analysis of RNA, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Short Protocols in Molecular Biology, fourth ed., Wiley and Sons, Inc., 1999 (Chapter 4).
[25] N.A. Smith, S.P. Singh, M.-B. Wang, P.A. Stoutjesdjk, A.G. Green, P.M. Waterhouse, Total silencing by intron-spliced hairpin RNAs, Nature 407 (2000) 319–320.
[26] S.J. Clough, A.F. Bent, Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*, Plant J. 16 (1998) 735–743.
[27] R. Welti, W. Li, M. Li, Y. Sang, H. Biesiada, H.-E. Zhou, C.B. Rajashekar, T.D. Williams, X. Wang, Profiling membrane lipids in plant stress responses, J. Biol. Chem. 277 (2002) 31994–32002.
[28] L. Kunst, A.L. Samuels, Biosynthesis and secretion of plant cuticular wax, Prog. Lipid Res. 42 (2003) 51–80.
[29] J.J. Thelen, J.B. Ohlrogge, Metabolic engineering of fatty acid biosynthesis in plants, Metab. Eng. 4 (2002) 12–21.
[30] H. Pinfield-Wells, E.L. Rylott, A.D. Gilday, S. Graham, K. Job, T.R. Larson, I.A. Graham, Sucrose rescues seedling establishment but not germination of Arabidopsis mutants disrupted in peroxisomal fatty acid catabolism, Plant J. 43 (2005) 861–872.
[31] M.X. Andersson, K.E. Larsson, H. Tjellstrom, C. Liljenberg, A.S. Sandelius, Phosphate-limited oat. The plasma membrane and the tonoplast as major targets for phospholipid-to-glycolipid replacement and stimulation of phospholipases in the plasma membrane, J. Biol. Chem. 280 (2005) 27578–27586.
[32] B. Eisenhaber, M. Wildpaner, C.J. Schultz, G.H. Borner, P. Dupree, F. Eisenhaber, Glycosylphosphatidylinositol lipid anchoring of plant proteins. Sensitive prediction from sequence-and genome-wide studies for Arabidopsis and rice, Plant Physiol. 133 (2003) 1691–1701.
[33] J.M. Shockey, M.S. Fulda, J.A. Browse, *Arabidopsis thaliana* contains nine long-chain acyl-coenzyme A synthetase genes that participate in fatty acid and glycerolipid metabolism, Plant Physiol. 129 (2002) 1710–1722.
[34] M. Fulda, J. Shockey, M. Werber, F.P. Wolter, E. Heinze, Two long-chain acyl-CoA synthetases from *Arabidopsis thaliana* involved in peroxisomal fatty acid β-oxidation, Plant J. 32 (2002) 93–103.
[35] M.-L. Chye, B.-Q. Huang, S.Y. Zee, Isolation of a gene encoding *Arabidopsis thaliana* membrane-associated acyl-CoA binding protein and immunolocalization of its gene product, Plant J. 18 (1999) 205–214.
[36] M. Metzner, K.P. Ruecknagel, J. Knudsen, G. Kuellertz, F. Mueller-Uri, B. Diettrich, Isolation and characterization of two acyl-CoA-binding proteins from proembryogenic masses of *Digitalis lanata* Ehrh, Planta 210 (2000) 683–685.
[37] G. Bonaventure, J.J. Salas, M.R. Pollard, J.B. Ohlrogge, Disruption of the FATB gene in *Arabidopsis thaliana* demonstrates an essential role of saturated fatty acids in plant growth, Plant Cell 15 (2003) 1020–1033.
[38] M.F. Miquel, D. James Jr., H. Donner, J. Browse, Arabidopsis requires polyunsaturated lipids for low-temperature survival, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 6208–6212.
[39] M.F. Miquel, J.A. Browse, High-oleate oilseeds fail to develop at low temperature, Plant Physiol. 106 (1994) 421–427.
[40] V. Eccleston, J.B. Ohlrogge, Expression of lauroyl-acyl carrier protein thioesterase in *Brassica napus* seeds induces pathways for both fatty acid oxidation and biosynthesis and implies a set-point for triacylglycerol accumulation, Plant Cell 10 (1998) 613–621.
[41] G. Bonaventure, X. Bao, J.B. Ohlrogge, M.R. Pollard, Metabolic responses to the reduction in palmitate caused by disruption of the FATB gene in Arabidopsis, Plant Physiol. 135 (2004) 1269–1279.
[42] D.V. Lynch, T.M. Dunn, An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function, New Phytol. 161 (2004) 677–702.
[43] T.M. Dunn, D.V. Lynch, L.V. Michaelson, J.A. Napier, A post-genomic approach to understanding sphingolipid metabolism in *Arabidopsis thaliana*, Ann. Bot. 93 (2004) 483–497.
[44] D.J. Sherrier, T.A. Prime, P. Dupree, Glycosylphosphatidylinositol-anchored cell-surface proteins from Arabidopsis, Electrophoresis 20 (1999) 2027–2035.
[45] F. Elortza, S. Mohammed, J. Bunkenborg, L.J. Foster, T.S. Nuhse, U. Brodbeck, S.C. Peck, O.N. Jensen, Modification-specific proteomics of plasma membrane proteins: identification and characterization of glycosylphosphatidylinositol-anchored proteins released upon phospholipase D treatment, J. Proteome Res. 5 (2006) 935–943.
[46] H. Zhu, W. Qian, X. Lu, D. Li, X. Liu, K. Liu, D. Wang, Expression patterns of purple acid phosphatase genes in Arabidopsis organs and functional analysis of AtPAP23 predominantly transcribed in flower, Plant Mol. Biol. 59 (2005) 581–594.
[47] C. Uhde-Stone, J. Liu, K.E. Zinn, D.L. Allan, C.P. Vance, Transgenic proteoid roots of white lupin: a vehicle for characterizing and silencing root genes involved in adaptation to P stress, Plant J. 44 (2005) 840–853.
[48] K.L. Johnson, K.A. Degnan, J. Ross Walker, G.C. Ingram, AtDEK1 is essential for specification of embryonic epidermal cell fate, Plant J. 44 (2005) 114–127.